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Evaluation of Enzyme Enantioselectivity in Supercritical Carbon Dioxide ($SC\text{-}CO_2$): Experimental Measurement and Thermodynamic Implications

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Thermodynamic analysis of the effect of solvent media on the enzyme-catalyzed conversion of acids and alcohols to a favored ester enantiomer requires precise measurement of the associated differential thermodynamic functions (Gibbs free energy, enthalpy, and entropy). The magnitude of these thermodynamic functions and the associated enantioselectivity (E) have been evaluated in the condensed liquid phase, however a comparison of these results for a model esterification reaction in supercritical fluid media has not been evaluated. In this study, 3-methyl-2-butanol was transesterified with vinyl octanoate in supercritical carbon dioxide ($SC\text{-}CO_2$) using Novozym 435 as the active lipase, yielding an excess of the R-enantiomer of 3-methyl-2-butyl octanoate. A novel stirred reaction vessel was constructed from a high pressure gauge snubber and placed in a commercial SFE instrument to facilitate measurement of E as a function of temperature in $SC\text{-}CO_2$. Using pressures between 14 - 22 MPa over a temperature range of 45 - 90° C, the thermodynamic functions and E values could be determined for the above reaction. Thermodynamic functions were evaluated via the Arrhenius dependence of the ln E. E was lower and the magnitude of the differential activation Gibbs free energy, enthalpy, and entropic term were found to all be decidedly less negative than the values displayed for these functions in liquid solvents over the same range of temperatures. This was found to be true when comparing results obtained using $SC\text{-}CO_2$ as a solvent with those obtained for running the same reaction in n-hexane, despite the similarity in solubility parameters for the two solvent media. The lower E value obtained for the model reaction in $SC\text{-}CO_2$ suggests that moderation should be applied in promoting the use of enzymatic conversions in supercritical fluid media, despite their many cited advantages.

Introduction

The coupling of enzymatic-initiated reactions with supercritical fluid media is appealing with respect to processing flexibility, environmental-compatibility, and utilization of benign synthesis conditions[1]. Enzymatic-base synthesis has been demonstrated particularly in supercritical carbon dioxide (SC-CO_2) [2-4], particularly for oleophilic substrates that are readily solvated in this dense gas medium. Such reactions involving the production of one chiral enantiomer over the other are of specific interest due to the products incorporation as biologically-active components, for flavor and sensory use, and as potential drug candidates[5,6].

The enzymatic kinetic resolution of a particular substrate can be measured by the enantio-selectivity parameter, E, given as:

$$E = \ln [(1 - ee_s)/(1 + ee_s/ee_p)] / \ln [(1 + ee_s)/(1 + ee_s/ee_p)] \quad (1)$$

where ee_s = enantiomeric excess of substrate, ee_p = enantiomeric excess of product, and c = conversion given as $c = ee_s/(ee_s + ee_p)$[7]. To optimize the enantiomeric excess of one particular product over the other requires detailed study of the influence of experimental conditions, including the role of solvent in achieving synthesis of particular chiral synthon. In this investigation, a hydrolase, Novozym 435, was tested in conjunction with SC-CO_2 over the pressure range of 14 - 22 MPa and temperature interval of 45 - 90° C, to achieve the transesterification of 3-methyl-2-butanol with vinyl octanoate. The reaction is driven by the tautomerisation of a by-product, acetaldehyde, to yield 3-methyl-2-butyl octanoate, as depicted in the reaction below in Figure 1.

The results of this thermodynamic study will be used for comparison purposes against data secured in previous studies in condensed phase organic solvents, such as decalin and n-hexane, which have larger molar volumes than compressed CO_2. Conditions were chosen so that SC-CO_2 might have a similar dielectric constant to n-hexane; however because of its smaller molecular size, (assuming the absence of fluid clustering), the achieved E values for the above model reaction may yield some insight as to the effectiveness of SC-CO_2 along with the reacting compounds, in solvating the actual catalytic site in the enzyme proper. By also studying the temperature dependence of the reaction, it is possible to assess the relative contributions of the activation enthalpy and entropy to

the free energy of the reaction, and the resultant enantioselectivity.

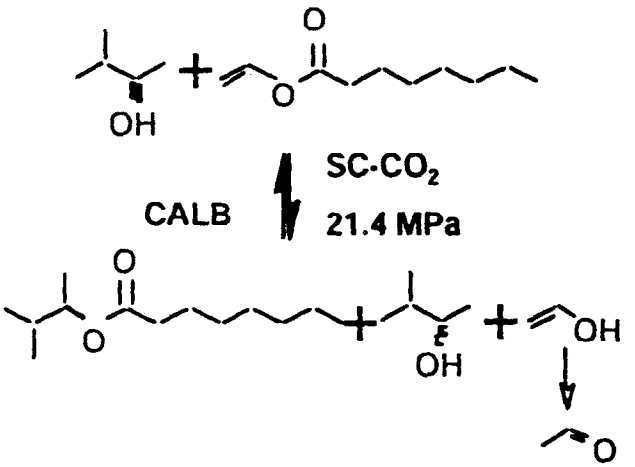

Figure 1. Enzymatically-catalyzed reaction between 3-methyl-2-butanol and vinyl octanoate in SC-CO_2.

Experimental Section

Novozym 435 was obtained from NOVO-Nordisk A/S in Denmark while vinyl octanoate was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The source for 3-methyl-2-butanol was Aldrich Chemical Co. (Milwaukee, WI). The ultra-high purity carbon dioxide employed in the experiments was SFE/SFC-grade CO_2 from Air Products (Allentown, PA).

The instrumentation used in performing the kinetic resolution studies is commercially-available equipment which was modified as follows for the described experiments. An Applied Separations Spe-ed unit=s (Applied Separations, Inc, Allentown, PA) liquid booster pump was to deliver the CO_2 up to the required density. In addition, the Spe-ed unit=s oven housed the reaction cell in a thermostated environment. Carbon dioxide was delivered through an on/off valve into the sampling loop (approx. 1 mL) of a Model 7125 Rheodyne injector (Rheodyne, Inc., Cotati, CA). Using the sampling valve arrangement as shown in Figure 2 permitting pressurized CO_2 to be delivered to the reaction cell, or subsequently reactants to be added to the cell, when appropriate.

The reaction cell consisted of a modified pressure gauge snubber (Part No. 30-31HF4, Chemiquip Products Co., Inc., New York, NY) that served as a small reaction cell. Basically, the snubber element was removed from the gauge snubber and a plug inserted at the bottom of the unit.

The modified gauge snubber proved to be an excellent reaction cell due to its flat bottom and sufficient internal volume which allowed for the inclusion of a small magnetic stirring bar in the cavity. Details of this assembly are given in Figure 3. The reaction cell was placed on top of a Lab-Line Magnestir (Part No. 1250), included in the oven proper, to facilitate mixing in the dense CO_2 phase. As shown by the schematic in Figure 2, valving and associated tubing were arranged to permit solvent flushing and collection of a sample from the reaction cell into a vial. Pressure and temperature were ascertained by using the sensors and readouts provided on the Spe-ed unit. An additional temperature indicator (Part No. 412A1A-OJC, Omega Engineering, Inc., Stamford, CT), with associated thermocouple, was used to monitor the temperature of the cell wall.

Fifty milligrams of Novozym 435, pre-equilibrated to a constant water activity level of 0.11 using a saturated solution of LiCl, were placed into the reaction cell. Dried vinyl octanoate, 420μL was then added to the reaction cell, and the mixture allowed to equilibrate for over 4 hours at the desired reaction temperature and a pressure of 13.8 MPa. The Rheodyne syringe loading sample injector was used to initiate the reaction by injecting 234 μL of 3-methyl-2-butanol and elevating the pressure to 21.4 MPa. Using this pressure at a temperature of 40° C provided a SC-CO_2 solubility parameter of 7.3 $(cal/mL)^{1/2}$, approximately that of n-hexane in the condensed state[8]. Samples were taken from the reaction cell at regular intervals commensurate with the rate of the reaction, using n-hexane (see Fig. 1) to wash any sample entrained in the valves/tubing into the collection vial. Carbon dioxide loss and pressure was reestablished by activating the Spe-ed unit=s booster pump.

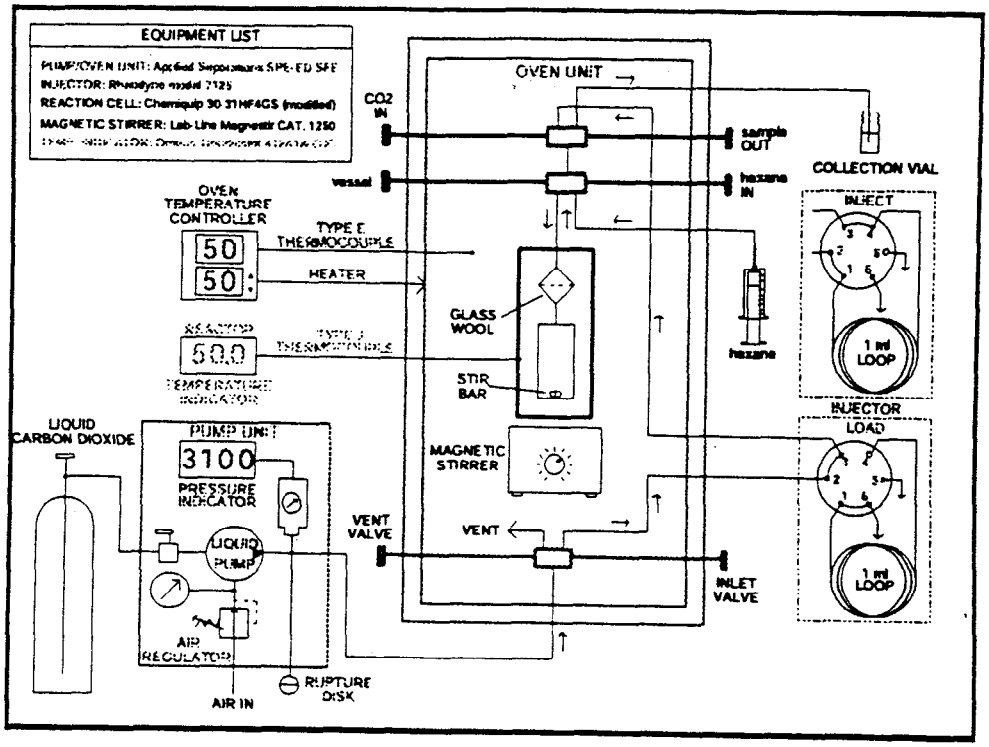

Figure 2. Schematic of experimental apparatus for enzyme enantioselectivity studies.

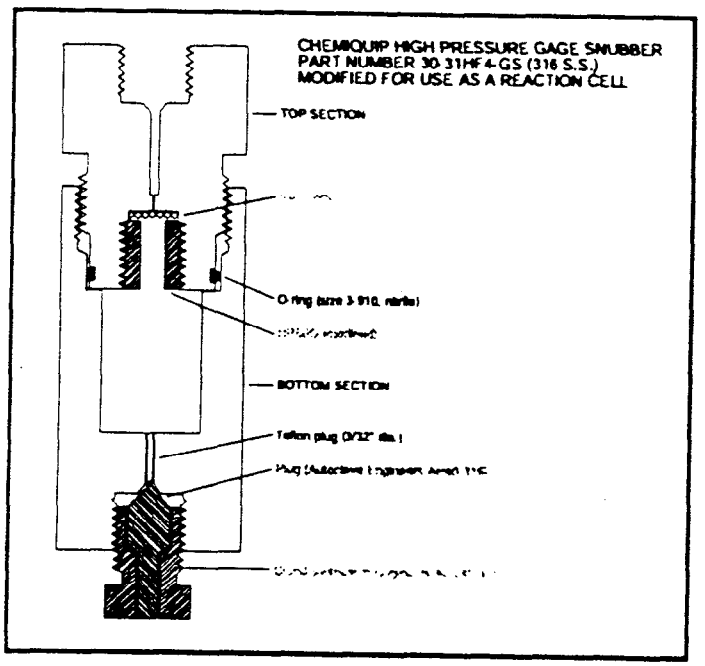

Figure 3. Cross-section of modified pressure snubber based reaction cell.

Analysis of the collected samples for enantiomeric excess of the 3-methyl-2-butanol, ee_s, and the product, 3-methyl-2-butyl octanoate (ee_p), was achieved using chiral capillary gas chromatography (GC). A Chirasil-Dex CB column from Chrompack, Inc. (The Netherlands) was used for this purpose. E was calculated as an average based on 4 - 14 samples ranging in conversion from 0-50 %.

Results and Discussion

Selectivity parameters for the kinetic resolution of 3-methyl-2-butanol were calculated using Equation 1. Calculation of differential activation thermodynamic functions was accomplished using the well known relationships given in Equations 2 and 3 as:

$$-RT \ln E = \Delta\Delta G^{I} = \Delta\Delta H^{I} - T\,\Delta\Delta S^{I} \qquad (2)$$

and

$$\ln E = \Delta\Delta H^{I}/R\,(1/T) + \Delta\Delta S^{I}/R \qquad (3)$$

assuming $\Delta\Delta H^{I}$ and $\Delta\Delta S^{I}$ are constant within the temperature range studied. If there is no exhibited enantioselectivity during the reaction, then E = 1, and one can solve for the racemic temperature, T_r, by rearranging Equation 2 as[9]:

$$T_r = \Delta\Delta H^{I}/\Delta\Delta S^{I} \qquad (4)$$

Hence, for temperatures lower than T_r, the entropic component will mitigate against E, and E will decrease with increasing temperature. Conversely, above T_r, the entropic component will influence E positively with an increase in temperature.

Prior studies have shown that Novozym 435, derived from *Candida antarctica*, is an excellent catalyst for the production of high purity enantiomers. The compensating effects of both the entropic and enthalpic components for reactions such as depicted in Figure 1, and the fact that T_r is higher than ambient temperature, makes pure synthon production feasible via enzymatic catalysis in heated liquid solvents and dense fluids. Figure 4 shows the results of the experimental measurement of E versus temperature for the reaction between 3-methyl-2-butanol and vinyl

octanoate (Figure 1) in SC-CO_2. As indicated by the results in Figure 4, enantiomeric enrichment of the ester product is best achieved at the lower temperatures over the range studied, ranging from approximately 225 at 45° C to a value of 100 at approximately 85° C. The influence of the thermodynamic parameters given in Equation 3 on the results for E will be discussed shortly.

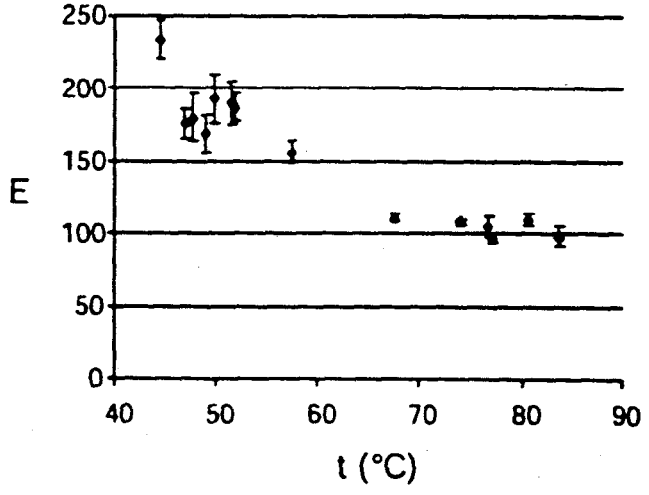

Figure 4. Variation in E with temperature (° C) for the transesterification of 3-methyl-2-butanol with vinyl octanoate in SC-CO_2.

It is interesting to compare the results obtained in Figure 4 with those determined in liquid solvent media over a similar temperature range[10]. Figure 5 shows the relationship between the ln E and the reciprocal of absolute temperature for three liquid solvents of varying molecular volume and the results obtained in SC-CO_2 from this study. The results for methylene chloride must be viewed as somewhat tentative since only three experimental determinations have been made to date. The relationship is approximately linear indicating an Arrhenius dependence of ln E on temperature. The larger magnitudes of E in the liquid solvents over the stated temperature range show that a greater enantioselectivity is achieved by running the reaction in a liquid solvent versus SC-CO_2. The ln E decreases with temperature for three liquid solvents examined, being of similar magnitude for decalin and n-hexane. However, E for the reaction in SC-CO_2 is considerably lower than in the three liquid solvents, indicating that SC-CO_2 is not always a preferred reaction medium for specific synthesis problems[10]. Nevertheless, the lower reaction selectivity in SC-CO_2 may be partially offset by the

environmentally-benign conditions that synthesis in SC-CO_2 offers, particularly in conjunction with the use of a naturally-derived enzyme moiety[11].

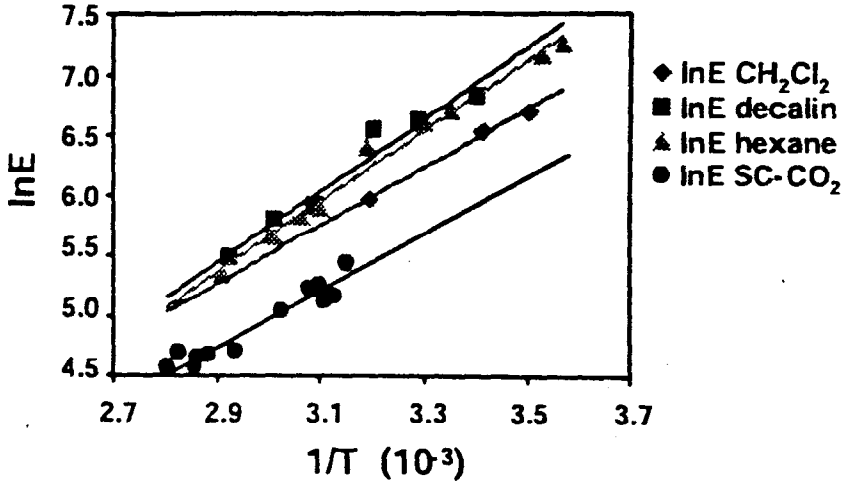

Figure 5. Variation in ln E vs. reciprocal of absolute temperature for enantioselective reaction between 3-methyl-2-butanol and vinyl octanoate in various solvent media.

The differential activation thermodynamic functions were also computed for the model reaction using Equation 3, and are presented in Figure 6. Both $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are negative for all the solvents considered, commensurate with a negative $\Delta\Delta G^{\ddagger}$ that supports the reactivity observed. In the case of n-hexane and decalin, the values of $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are approximately the same magnitude, however there is an increase in $\Delta\Delta H^{\ddagger}$ (more positive) when methylene chloride or SC-CO_2 is used as the reaction medium. This $\Delta\Delta H^{\ddagger}$ value suggests a steric-based discrimination between the enantiomers at the active site in the enzyme that is dependent on the nature of the solvent. Similarly, $\Delta\Delta S^{\ddagger}$ values for the reaction run in methylene chloride and SC-CO_2, respectively, are approximately 3 kJ/mole greater (more positive) than those exhibited for the reaction when run in the two hydrocarbon solvents. This would seem to argue that the smaller solvents facilitate either a positive or negative change in the entropy of the R or S enantiomer, respectively. This inhibitory effect observed in the smaller solvents would seem to suggest a mechanism whereby the solvent molecules are capable of occupying more space around the active site in the enzyme, thereby blocking the kinetic transformation of one enantiomer over the other[12]. This could change as the molar volume of SC-CO_2 changes by varying the pressure or temperature, certainly one of the

advantages to employing SC-CO_2 as a solvent medium for enzymatic-catalyzed reactions.

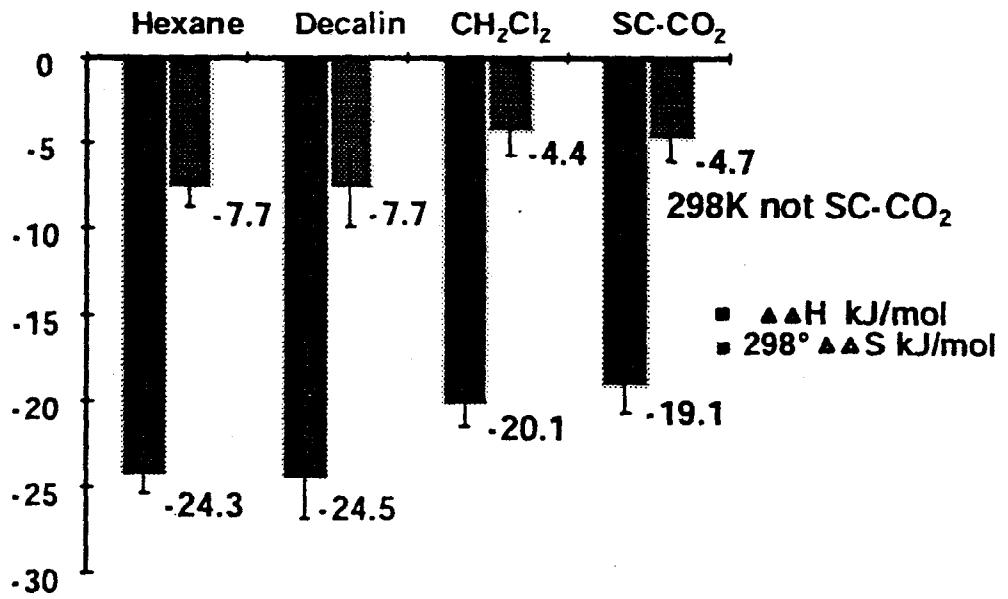

Figure 6. Differential activation $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ values in different reaction solvents.

Conclusions

The reported study provides fundamental knowledge as to the factors influencing enantioselectivity in an enzyme-catalyzed model reaction in SC-CO_2. In contrast to similar studies of the same model reaction in organic solvents, the determined E values in SC-CO_2 are smaller in magnitude than those found for the same transesterification reaction in liquid media; suggesting that caution should be applied in advocating the use of supercritical fluids for attaining high E values. Differential activation thermodynamic functions, $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$, tend to also be more positive in SC-CO_2 for the reaction between 3-methyl-2-butanol and vinyl octanoate, than those recorded in liquid solvents having a similar solubility parameter (n-hexane). The described experimental apparatus has proved facile in the determination of the above data, and further studies are continuing on pressurized liquid solvent media, as well as CO_2 dissolved in compressed n-hexane to further our basic understanding.

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